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Peptides with Carboxyl-Terminal Sequence of Alanine–Proline: Detection by a Human Monoclonal Antibody

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ABSTRACT

A human B lymphoblastoid cell line JWCI-L94 secretes an IgM human monoclonal antibody (HuMAb) that reacts with human melanoma cell lines, M14 and M12. To identify the antigenic epitope of this antibody, we screened λgt11 expression libraries constructed from M14 and M12. A total of 12 immunoreactive clones were isolated, and their DNA sequences were determined. The only sequence shared by all these clones was alanine–proline (A–P) at the carboxyl (C) terminal. HuMAb L94 reacted not only with C-terminal A–P-containing fusion proteins, but also with the synthetic dipeptide A–P. None of the peptides containing A–P internally or amino terminally reacted to HuMAb L94. Proline or alanine alone had no ability to bind to HuMAb L94. When alanine was replaced by glycine (G–P) or proline (P–P), the binding activity of these peptides was similar to that of A–P. On the other hand, when alanine was replaced by serine, valine, leucine, glutamine, lysine, methionine, phenylalanine, or hydroxyl proline, the resulting peptide completely lost the antigenic activity of HuMAb L94. These results demonstrate that HuMAb L94 recognizes C-terminal A–P, G–P, or P–P, and that a human antibody can recognize peptides as small as a two-amino acid residue.

INTRODUCTION

BY THE USE of the Epstein–Barr virus (EBV), we have established a number of human B lymphoblastoid cell lines that produce human monoclonal antibodies (HuMAbs) to melanoma-associated antigens.[1] Chemical analysis of antigen epitopes for three such HuMAbs has identified the antigens as glycolipid antigens: ganglioside GD_2, GM_2, and GM_3, respectively.[2–4] Epitopes recognized by these HuMAbs consist of only two or three terminal sugar residues of these glycolipids. In the present study we report a new HuMAb that has specificity for a peptide antigen, the epitope of which consists of a C-terminal two-amino acid residue. To our knowledge this is the smallest peptide epitope reported that is recognized by a human antibody.

MATERIALS AND METHODS

Human tumor cell lines

Cultured human melanoma cell lines, M12 and M14, were maintained in RPMI 1640 medium supplemented with 5% fetal calf serum (FCS), penicillin (100 U/ml), and streptomycin (100 μg/ml).

Vectors, bacterial strains, cDNA library, and oligonucleotides

The bacteriophage λgt11, *Escherichia coli* cell strains Y1089 and Y1090, and the plasmid Bluescript (Promega, Madison, WI) were purchased from Stratagene (San Diego, CA). Expression cDNA libraries of human melanoma cell lines M14 and M12 were constructed at the *Eco*RI site of λgt11, using the oligo(dT) priming method (Invitrogen, San Diego, CA). The cDNAs cloned in λgt11 were subcloned into plasmid Bluescript and analyzed for their sequences, using a modified dideoxy chain termination method (U.S. Biochemical, Cleveland, OH).

Human monoclonal antibody JWCI-L94 (HuMAb L94)

An IgM human monoclonal antibody, HuMAb L94, was established from the peripheral blood lymphocytes (PBLs) of a melanoma patient using the EBV transformation technique as previously described.[1] Melanoma cell lines UCLA M14 and

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M12 were used to monitor the antibody secreted in the spent tissue culture medium, using immunohistochemical assays. Purification of HuMAb L94 from the spent medium was performed as previously described.[5] To determine an approximate molecular size of the antigen, these cell lines were tested by sodium dodecyl sulfide-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting analysis with HuMAb L94, but produced no visible band. This indicates either that the antigenicity was altered, unexpressed, or became cryptic during the SDS-PAGE procedure, or that the antigenic epitope resides in a glycolipid. The latter possibility, however, was unlikely because neither acidic nor neutral glycolipid fractions purified from these cell lines exhibited antigenicity.

Screening of melanoma cell cDNA expression library with HuMAb L94

Screening of λgt11 cDNA library constructed from melanoma cell lines M14 and M12 was performed as previously described.[6] Briefly, nitrocellulose filters were exposed to a melanoma cDNA expression library. Nitrocellulose filter lifts were reacted sequentially with HuMAb L94 and a goat anti-human IgM (μ chain specific) antibody conjugated with peroxidase (Boehringer Mannheim, Indianapolis, IN). The antigen–antibody complexes were visualized with the chromogenic substrate 4-chloro-1-naphthol.

Immunological assays

SDS-PAGE followed by Western blotting were carried out as previously described.[7,8] Peptides, glycine–proline (G–P), alanine–proline (A–P), alanine–proline–amide (A–P–amide), β-alanine–proline (β-A–P), alanine–proline–glycine (A–P–G), alanine–proline–alanine (A–P–A), proline–proline (P–P), lysine–proline (K–P), serine–proline (S–P), methionine–proline (M–P), leucine–proline (L–P), glutamic acid–proline (E–P), glycine–hydroxyproline–proline (G–hP–P), and valine–proline (V–P) were purchased from Bachem Bioscience, Inc. (Philadelphia, PA), Sigma (St. Louis, MO), and Novabiochem USA (La Jolla, CA). The solid-phase enzyme-linked immunosorbent assay (ELISA) was used to test the binding of HuMAb L94 to peptide antigens. Peptide (1 μg/well) was coated on the Reacti-Bind™ plate (Pierce, Rockford, IL) and left overnight at 4°C. The ELISA was performed using a goat anti-human IgM (μ chain specific) conjugated with peroxidase. Data were analyzed using a plate reader and Softmax (Molecular Devices, Menlo Park, CA). An inhibition ELISA was performed to examine cross-reactivity of HuMAb L94 with several synthetic antigens (10 mg of peptide/μg of HuMAb). Preincubated HuMAb L94 with peptides was tested using A–P-coated plates.

In situ *hybridization*

A synthetic oligodeoxynucleotide sequence complementary to mRNA for peptide 707 (GTC GCA GCC CTT GCT CGA GAC GCC CCG) was end labeled with digoxigenin and used to detect mRNA in a panel of cells. Cells fixed on glass slides were prehybridized for 1 hr at 42°C in a solution containing deionized formamide, 20× standard saline citrate, Denhardt's solution, heat-denatured sheared herring sperm DNA, yeast transfer RNA, and dextran sulfate. The 707 antisense probe (Molecular Biology Institute, UCLA School of Medicine, Los Angeles, CA) was tail labeled with digoxigenin-11-dUTP (Boehringer Mannheim) using the DNA tailing kit (Boehringer Mannheim). A negative control was carried out by using the 707 sense probe, which was complementary to the test (antisense) probe, in the reaction. A 27-bp oligonucleotide probe specific to human fibroblast β-actin (Clontech Laboratories, Inc., Palo Alto, CA) was used for a positive control. A digoxigenin-labeled probe was placed on the cells and incubated at 42°C overnight. Cells containing 707 mRNA were detected using the Genius nonradioactive nucleic acid detection kit (Boehringer Mannheim). Slides were incubated with 2% normal sheep serum and 0.3% Triton X-100 at room temperature for 30 min. Anti-digoxigenin antibody was applied to the cells for 3 hr at room temperature. A solution containing nitroblue tetrazolium, X-phosphate solution, and levamisole was placed on the cells at room temperature until they developed a satisfactory color (2–5 hr).

RESULTS

To isolate the cDNA encoding epitope for HuMAb L94, we first screened cDNA library constructed from M14 melanoma cell line at the *Eco*RI site of λgt11. Seven clones detected by the antibody were isolated. All clones were then expressed in *E. coli* Y1090 cells, using β-galactosidase as a fusion partner. The expressed proteins were analyzed by SDS-PAGE and Western blotting with HuMAb L94 (Fig. 1). All of the fused proteins showed clear bands in both SDS-PAGE and Western blotting, but β-galactosidase alone failed in reactivity. This result indicates that HuMAb L94 bound specifically to the sequence derived from the cDNA insert, but not to β-galactosidase. These clones were subcloned into plasmid Bluescript at the *Eco*RI site and their DNA sequences were analyzed by dideoxy termination methods. The DNA sequences and their deduced amino acid sequences are shown in Fig. 2. Five of seven clones had independent sequences. Clones 711 and 807 had the same DNA sequence. Clone 703B is part of clone 707. Although the length of cDNA inserts ranged from 0.6 to 2.0 kb, their protein coding regions were much shorter than those expected from the cDNA length (Fig. 2). This result was consistent with the molecular

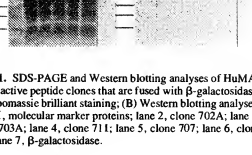

FIG. 1. SDS-PAGE and Western blotting analyses of HuMAb L94 reactive peptide clones that are fused with β-galactosidase. (A) Coomassie brilliant staining; (B) Western blotting analyses. Lane 1, molecular marker proteins; lane 2, clone 702A; lane 3, clone 703A; lane 4, clone 711; lane 5, clone 707; lane 6, clone 808; lane 7, β-galactosidase.

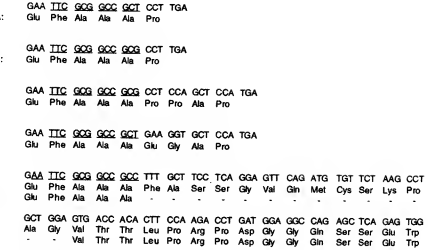

FIG. 2. cDNA sequences of HuMAb reactive clones and amino acid sequences deduced from their cDNA sequences. Underlining indicates the linker sequence.

weights of immunoreactive fused proteins, whic mobilized at a position similar to that of β-galactosidase (Fig. 1).

All of the amino acid sequences deduced from the DNA sequence of each clone had A–P at their C-terminals. In particular, the fusion proteins of clones 702A and 703A were composed only of β-galactosidase, the peptide derived from linker sequence, and A–P derived from the cDNA insert. In addition to the M14 cDNA library, we screened the cDNA library constructed from M12, and we isolated five clones. All five clones also had a C-terminal A–P sequence (data not shown). To determine if dipeptide A–P formed the epitope for HuMAb L94, synthetic peptide A–P was examined for its binding to the antibody by ELISA. As shown in Fig. 3A, HuMAb L94 bound to dipeptide A–P in a dose-dependent manner. To examine whether or not the position of the dipeptide A–P must be at the C-terminal, a series of synthetic oligopeptides were tested by the peptide ELISA. As shown in Fig. 3B (columns 2–4), the addition of Gly, Ala, or Ala–Ala to the C-terminal of A–P abolished the binding ability of HuMAb L94. The reactivity of amino (N)-terminal A–P was tested by Western blot analysis using recombinant human interleukin 1β (IL-1β) as an antigen source, of which the N-terminal is A–P. The protein showed negative reactivity by HuMAb L94 (data not shown). These data demonstrate that C-terminal localization of A–P was essential to exhibit the antigenicity for HuMAb L94. In the next series of experiments, we tested a variety of dipeptides for cross-reactivity with HuMAb L94. Substitution of C-terminal Pro by Thr, Tyr, or 4-hydroxy-Pro abolished the binding ability completely (Fig. 3B, columns 6–8). Substitution with Pro-amide significantly reduced the binding ability (Fig. 3B, column 5). These results indicated that a C-terminal Pro residue was essential for recognition by the HuMAb L94. Subsequently, the influence of the adjacent amino acid residue of the C-terminal on the antibody binding was tested. When Ala was substituted with β-Ala, Gly, or Gly–Pro (Fig. 3B, columns 9, 10, and 19), the antigenicity was found to be intact. However, antibody binding became completely negative when Ala was substituted with hydroxy-Pro, Ser, Val, Glu, Lys, Met, Leu, and Phe (Fig. 3B, columns 11–18). The amino acid residue of this second position seemed to be restricted to the smaller size of its side chain. The peptide Pro–Pro exhibited low binding ability, probably indicating that the maleic anhydride of the Reacti-Bind plate (Pierce) scarcely reacted with the amino group of the proline residue. The immunopositive peptides in Fig. 3B were employed to test their cross-reactivity by ELISA inhibition assay. As shown in Fig. 3C, the binding of HuMAb L94 to A–P was completely inhibited by A–P, G–P, and P–P and was significantly inhibited by β-A–P and A–P–amide, but not by proline alone or A–P–G. These data suggest that all three peptides proved to be immunoreactive by direct peptide ELISA (A–P, G–P, and P–P) have a cross-reactive epitope for HuMAb L94.

The expression of A–P antigen or cross-reactive antigens on the surface of melanoma cells was tested by an absorption assay. After HuMAb L94 was absorbed with an antigenic melanoma cell line, M14, the binding of HuMAb L94 to β-galactosidase-fused protein 711 was reduced significantly. Antibody activity was not absorbed with nonantigenic melanoma cell line M15 in the same assay (Fig. 4). Furthermore, preincubation of HuMAb L94 with dipeptide A–P reduced IA activity against M14 cells. Treatment of target M14 cells with protease such as trypsin and carboxypeptidases Y and P (Boehringer Mannheim) significantly reduced the immuno-adherence (IA) activity of HuMAb L94 (data not shown). These data indicate that C-terminal A–P or a cross-reactive sequence is expressed on the cell surface of melanoma M14.

In situ hybridization for mRNA of one of the cloned peptide 707 was performed on nine human melanoma cell lines, nine other histological types of human cancer cell lines, and normal lymphocytes. As shown in Table 1, 100% of melanoma,

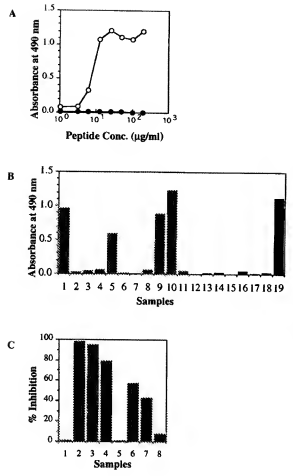

FIG. 3. Binding of HuMAb L94 to peptides in a solid-phase ELISA. (A) Binding of HuMAb L94 to synthetic dipeptide A–P. ELISA plate (Reacti-Bind™; Pierce) was coated with two fold serially diluted dipeptide A–P. HuMAb L94 was added to each well, and bound HuMAb was detected by a peroxidase-labeled goat anti-human IgM (μ). (B) Binding of HuMAb L94 to a variety of synthetic peptides. ELISA plate was coated with 1 μg of peptide/well. Peptides were (1) Ala–Pro; (2) Ala–Pro–Pro–Ala–Pro–Ala–Ala; (3) Ala–Pro–Gly; (4) Ala–Pro–Ala; (5) Ala–Pro–amide; (6) Ala–Thr; (7) Ala–Tyr; (8) Gly–hPro; (9) Gly–Pro; (10) Gly–Pro–Pro; (11) Gly–hPro–Pro; (12) Ser–Pro; (13) Val–Pro; (14) Glu–Pro; (15) Lys–Pro; (16) Met–Pro; (17) Leu–Pro; (18) Phe–Pro; and (19) β-Ala–Pro. (C) Binding inhibition of HuMAb L94 by peptides to assess the cross-reactivity of HuMAb L94. Before the ELISA, HuMAb L94 was mixed with the following peptides (10 mg/ml): (1) PBS(−); (2) Ala–Pro; (3) Gly–Pro; (4) Pro–Pro; (5) Pro; (6) β-Ala–Pro; (7) Ala–Pro–amide; and (8) Ala–Pro–Gly. The residual antibody-binding activity was tested using an ELISA plate coated with Ala–Pro peptide (1 μg/well).

including those negative for membrane antigen expression, such as M15, were positive for mRNA; however, only one-third of other types of cancer (one colon cancer, one gastric cancer, and one breast cancer cell line) were positive. Monocytes, B lymphocytes, and T lymphocytes obtained from peripheral blood lymphocytes were all negative.

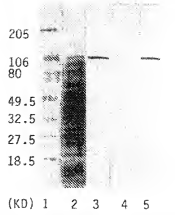

FIG. 4. Absorption of HuMAb L94 with intact melanoma cells. The recombinant phage lysogens (clone 711) were cultured at 30°C, and protein expression was induced by isopropyl thiogalactosidase (IPTG) treatment and temperature shift to 42°C. The extract from lysogen was used to detect the reactivities of absorbed HuMAb L94 on SDS-PAGE Western blotting. Lane 1, molecular weight standard proteins were stained with Coomassie Brilliant Blue; lane 2, clone 711 proteins were stained with Coomassie Brilliant Blue, lanes 3–5 were stained using Western blotting; primary antibody (HuMAb L94) was preincubated with PBS (lane 3), the antigenic M14 melanoma cell line (3×10^7 cells) (lane 4), and an antigen-negative M15 melanoma cell line (3×10^7 cells) overnight at 4°C (lane 5). The resultant antibodies were examined by using SDS-PAGE Western blotting of clone 711.

DISCUSSION

Screening of an expression of cDNA library of cultured human melanoma cell M14 with HuMAb resulted in the isolation of five independent clones of seven clones. Each sequence was compared with reported sequences, using PC/GENE software (IntelliGenetics, Inc., Mountain View, CA). No homologous proteins of clones 703A, 707, and 811 were found. Clone 702A is a part of the sequence of the 3′-untranslated region of human γ cytoskeletal actin,[9] and clone 808 is a part of the coding region of human melanocyte specific gene PMEL 17,[10] although its reading frame is different from the original frame. We also isolated five immunoreactive clones from a cDNA library constructed from melanoma cell line M12. All five of these clones had C-terminal A–P. It is puzzling that, while the isolated clones have only A–P sequence at their C-terminals, HuMAb L94 bound not only to A–P, but also to dipeptides G–P and P–P. Presumably, C-terminal A–P may have expressed the strong antigenicity after a conformational constraint by interacting with some part of the fusion partner β-galactosidase. Alternatively, C-terminal G–P and P–P may not interact suitably to exert antigenicity for HuMAb L94 during the cloning step.

Two mechanisms may account for the expression of this epitope by the melanoma cell: (1) a known or unknown protein with C-terminal A–P expressed on or in the cell; or (2) a known

TABLE 1. EXPRESSION OF MRNA FOR #707 PEPTIDE BY HUMAN CANCER CELL LINES AND NORMAL LYMPHOCYTES

Cell lines	*Histologic types*	In Situ *Hybridization*[a]
M10	Melanoma	+
M12	Melanoma	+
M14	Melanoma	+
M15	Melanoma	+
M24	Melanoma	+
M25	Melanoma	+
M101	Melanoma	+
M111	Melanoma	+
M12	Melanoma	+
K562	Erythroleukemia	–
SHN	Neuroblastoma	–
SW480	Colon Cancer	–
SW48	Colon Cancer	+
MKN45	Gastric Cancer	+
MNK28	Gastric Cancer	–
B645	Breast Cancer	+
L130	Lung Cancer	–
L135	Lung Cancer	–
Peripheral Blood Lymphocyte	Monocytes	–
	B cells	–
	T cells	–

[a]mRNA level for #707 peptide was analyzed using, digoxigenin-labeled complementary oligonucleotide probes.

or unknown protein that has A–P within the entire sequence is processed by a proteinase(s), which is specifically activated in neoplastic cells such as melanoma. Seven different human proteins with C-terminal A–P have been reported: type I membrane protein, T cell differentiation antigen CD6,[11] leukosialin CD43,[12] extracellular signal-regulated kinase 1,[13] DNA-binding protein, interferon regulatory factor,[14] plasma protein, protein C,[15] protein alkaline phosphatase precursor,[16] and endothelin-3 precursor.[17] Although we have not tested the reactivity of these known proteins with HuMAb L94, it is most likely that the results would be positive. In fact, we purchased two different synthetic peptides with C-terminal A–P from Sigma (bradykinin potentiator B and pEKWAP) and tested the HuMAb reactivity. Strong antigenicity was obtained from both of these commercially available peptides. In support of the second hypothesis, it has been reported that certain enzymes could be activated during malignant transformation such as those for fibronectin[18] and proteoglycan.[19] These proteins are cleaved by a protease and the resultant new N- and C-terminals of the proteins have different properties to be recognized by an antibody. Prolylendopeptidase (EC 3.4.21.26) is typical of this type of enzyme, because it specifically cleaves the C-terminal side of the Pro–X peptide bond.[20]

Antigenic determinants that include the A–P sequence within peptides have been reported. The monoclonal antibodies against carcinoma-associated epithelial mucins bind to peptides containing the sequence RPAP,[21,22] and the monoclonal antibodies against the human B cell differentiation marker CD24 recognize the LAP sequence.[23] The antigenicity of neither of these monoclonal antibodies depends on the position of A–P at its C-terminal, whereas the antigenicity of HuMAb L94 occurs only at the C-terminal A–P sequence.

There is no consensus regarding the smallest moiety that can function as a complete antigenic determinant. Most estimates for the minimum number of sequential amino acids have been around six. A direct enzyme-linked immunosorbent assay has detected dipeptide epitopes for certain monoclonal antibodies.[21,24,25] These previous studies used murine monoclonal antibodies raised against peptide or whole cell. Although the parent protein molecule still remains to be defined, our antibody is unique, because, to the best of our knowledge, HuMAb L94 is the only human monoclonal antibody reported of which the antigenic determinant is only two amino acids.

The antigen-binding region of HuMAb L94 may form a narrow binding pocket, the bottom part of which fits in the C-terminal part of dipeptide A–P. Proline alone did not show any interaction with HuMAb L94. Proline can go into the binding pocket, but free amino acid does not have the correct conformation or interaction to bind with HuMAb L94. The adjacent residue Ala can be replaced by Gly or Pro. The size of the side chain of Gly and Pro is similar (or smaller) to that of Ala. Residues that have a much more bulky side chain than Ala, such as Ser, Val, and Glu, may not, however, be able to enter the binding pocket because of their steric hindrance.

Our laboratory is currently investigating the biological and clinical significance of C-terminal A–P, as well as tumor-associated proteins with C-terminal A–P. Using the cloned sequence 707, we have demonstrated that the C-terminal A–P sequence played an important role in the induction of cytotoxic T cell line (CTL) specific to melanoma cells.[26]

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